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Downstream of the *dbl-1* TGFβ Signaling Pathway

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In *Caenorhabditis elegans*, two well-characterized TGFβ signaling cascades have been identified: the *Sma*ll/*M*ale tail *ab*normal (Sma/Mab) and *Da*uer *f*ormation (Daf) pathways. The Sma/Mab pathway regulates body size morphogenesis and male tail development. The ligand of the pathway, *dbl-1*, transmits its signal through two receptor serine threonine kinases, *daf-4* and *sma-6*, which in turn regulate the activity of the Smads, *sma-2*, *sma-3*, and *sma-4*. In general, Smads have been shown to both positively and negatively regulate the transcriptional activity of downstream target genes in various organisms. In *C. elegans*, however, target genes have remained elusive. We have cloned and characterized *lon-1*, a gene with homology to the *c*ysteine-*ri*ch *s*ecretory *p*rotein (CRISP) family of proteins. *lon-1* regulates body size morphogenesis, but does not affect male tail development. *lon-1* is expressed in hypodermal tissues, which is the focus of body size determination, similar to *sma-2*, *sma-4*, and *sma-6*. Using genetic methods, we show that *lon-1* lies downstream of the Sma/Mab signaling cascade and demonstrate that *lon-1* mRNA levels are up-regulated in *sma-6*-null mutant animals. This provides evidence that *lon-1* is negatively regulated by Sma/Mab pathway signaling. Taken together, these data identify *lon-1* as a novel downstream target gene of the *dbl-1* TGFβ-like signaling pathway. © 2002 Elsevier Science (USA)

***Key Words:* *Caenorhabditis elegans*; body size; TGFβ; signal transduction; *dbl-1*; CRISP.**

INTRODUCTION

Transforming growth factor β (TGFβ) regulates many cellular processes, including proliferation, differentiation, development, and apoptosis. The TGFβ superfamily includes the TGFβs, activins, and bone morphogenetic proteins (BMPs). These ligands transmit their signal upon binding to a type II receptor serine threonine kinase (RSK), which in turn phosphorylates and activates a type I RSK. The active type I RSK transmits the signal to downstream molecules called Smads. The receptor-regulated Smads (R-Smads) are phosphorylated by the type I RSK, which allows them to form a complex with the common Smads (co-Smads) and translocate to the nucleus. Once in the nucleus, the Smads bind DNA and act as transcriptional regulators (Heldin *et al.*, 1997; Massagué, 1998; Patterson and Padgett, 2000; Whitman, 1998). An intense area of investigation is to identify downstream target genes that mediate the diverse functions of TGFβ.

In *Caenorhabditis elegans*, there are two well-studied TGFβ-like signaling pathways, the Sma/Mab (*Sma*ll/*M*ale tail *ab*normal) pathway and the Daf (*da*uer *f*ormation) pathway (reviewed in Patterson and Padgett, 2000). The Sma/Mab pathway regulates body size formation and male tail development (Morita *et al.*, 1999; Savage *et al.*, 1996; Suzuki *et al.*, 1999). The dauer pathway regulates the formation of an alternative larval stage when there is an excessive population of worms and/or food is scarce (Riddle and Albert, 1997). Entry into the dauer stage allows for survival until resources are plentiful again. Both of these pathways utilize a common type II RSK, *daf-4* (Krishna *et al.*, 1999). *daf-4 (lf)* animals possess the phenotypes seen in both Daf and Sma/Mab pathway mutants (Estevez *et al.*, 1993). These animals are dauer constitutive (develop into dauer larvae when food is plentiful), are small in body size, and males possess malformed tail rays and spicules.

Mutations in any of the known Sma/Mab pathway components result in animals that are about 30% smaller in

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body size than wild-type animals but, unlike *daf-4*, have no dauer phenotypes on their own (Estevez *et al.*, 1993; Krishna *et al.*, 1999; Morita *et al.*, 1999; Savage *et al.*, 1996; Suzuki *et al.*, 1999). In the tail of male mutant animals, ray fusions are seen between rays 4 and 5, 6 and 7, as well as 8 and 9 (Krishna *et al.*, 1999; Savage *et al.*, 1996; Savage-Dunn *et al.*, 2000). In addition, the copulatory spicules are crumpled. These male tail defects prevent the male from mating. The Sma/Mab ligand, *dbl-1*, and the RSKs, *daf-4* and *sma-6*, transmit the TGFβ signal to the R-Smads. However, in *C. elegans*, three Smads, *sma-2*, *sma-3*, and *sma-4*, are required for proper TGFβ signaling as opposed to the two interacting Smads seen in *Drosophila* and mammalian systems. Once activated, the Smads translocate to the nucleus and alter target gene transcription. In *C. elegans*, very little is known about the downstream Sma/Mab pathway targets.

In efforts to elucidate the downstream cellular outputs of Sma/Mab pathway signaling, we examined long body sized animals (Lon). Since overexpression of some Sma/Mab pathway components leads to Lon body size, we reasoned that *lon-1*, which also affects body size, might be involved in Sma/Mab pathway signaling (Morita *et al.*, 1999; Suzuki *et al.*, 1999). We find through epistatic analyses that *lon-1* functions downstream of the Sma/Mab pathway components in regards to regulating body size morphogenesis but not male tail development. We have cloned *lon-1* and find that it encodes a protein with homology to several secreted molecules of the *c*ysteine-*r*ich *s*ecretory *p*rotein (CRISP) family (Kratzschmar *et al.*, 1996). Although several diverse molecules comprise the CRISP family, such as sperm-coating glycoproteins, venom allergen 5, and PR-1 plant pathogenesis proteins, little is understood about how they function. We find that *lon-1* is expressed in the hypodermis and intestine, similar to the expression patterns seen for known Sma/Mab pathway components. In addition, we provide evidence that *lon-1* function is required in the hypodermis, and not the intestine, to regulate proper body size formation. Examination of *lon-1* mRNA levels shows that mutations in Sma/Mab pathway signaling negatively regulate *lon-1* expression. Therefore, we conclude that *lon-1* is a Sma/Mab pathway target gene.

MATERIALS AND METHODS

C. elegans Strains

All strains were grown at 20°C and maintained by using standard laboratory procedures (Brenner, 1974). The Bristol strain, N2, was used as wild-type. *sma-6(wk7)* and *lon-1(wk50)* were used in epistatic analyses. The triple mutant *lon-1(e185) unc-119(e2498); him-5(e1490)* was used for cosmid rescue. *lon-1(e185)*, *lon-1(n1130)*, *lon-1(ct411)*, *lon-1(wk49)*, *lon-1(wk50)*, *sma-6(wk7)*, and N2 were used in developmental time course experiments. Genomic DNA from *lon-1(wk50)*, *lon-1(e185)*, and *lon-1(n1130)* was sequenced to identify molecular lesions. RNA from a *dbl-1* overexpressing strain, *ctIs40* [*pTG96 (sur-5::gfp)*, *ZC421(dbl-1)*], (Suzuki *et al.*, 1999) was used in Northern blot analyses described below.

Isolation of lon-1 Alleles

The canonical allele *lon-1(e185)* was isolated by Brenner (1974). We isolated additional *lon-1* mutants from an ethyl methanesulfonate (EMS)-induced mutant F2 screen (unpublished data). *lon-1(wk49)*, *lon-1(wk50)*, and *lon-1(wk51)* were identified based on the long body size phenotype of mutagenized N2 animals and their failure to complement *lon-1(e185)*.

Cloning of lon-1

lon-1 was mapped to linkage group III between *dpy-17* and *sma-2* by Brenner (1974). Six cosmids corresponding to the *lon-1* interval were tested for transformation rescue by coinjecting with marker plasmid containing wild-type *unc-119* into *lon-1(e185) unc-119(e2498); him-5(e1490)* triple mutant animals. Cosmid F48E8 conferred rescue. A genomic lambda library was screened in order to isolate phage clones spanning the entire rescuing cosmid. Subsequently, two of these phage clones conferred rescue, identifying F48E8.1 as the *lon-1* open reading frame.

We isolated a cDNA, B1.11, from a library (R. Barstead, personal communication). We also obtained the cDNA yk298h6 from the *C. elegans* cDNA Project (Y. Kohara, National Institute of Genetics). Each cDNA was sequenced and compared with the genomic sequence from cosmid F48E8. Both cDNAs contain six exons. However, the first five exons are identical, while the sixth exon differs (Figs. 1B and 1C).

To sequence mutant alleles, genomic DNA was isolated from homozygous *lon-1(wk50)*, *lon-1(e185)*, and *lon-1(n1130)* animals. To eliminate PCR errors, two independent samples for each primer set and each worm strain were generated. The *lon-1* region was amplified by PCR using primer sets that spanned the genomic region of *lon-1* (CGGCGACTAGTCGGCTATATTGCAGATAC, CGGCGCTCGAGGCAAAGCCAAAGGAGACA; CGGCGACTAGTTTTGAGTTTCTTGCTAAC, CGGCGCTCGAGTTAAAACATAAACGGATC). All fragments were digested with *Spe*I and *Xho*I, cloned into pBluescript SK+, and sequenced.

Body Size Measurements

Images were taken of animals at L4 stage and 48 h later at a magnification of 3.5× using a Nikon SMZ-U dissecting microscope. The computer program used to capture the images was Strata Video Shop (Strata Inc.) with screen dimensions 680 × 460 pixels. Once images were recorded, perimeter analysis on at least 19 animals was performed by using Image Pro Plus software (Mediacybernetics).

Developmental Time Course Analysis

In order to obtain a synchronous population, gravid animals were treated with a hypochlorite/NaOH solution to remove all animals while leaving the eggs unaffected. Eggs were then placed into 10 ml of M9 for 24 h and allowed to hatch at room temperature. L1 animals were plated onto OP50 seeded agar plates (time zero) and allowed to develop at 20°C. Pictures were taken of animals at 24-h intervals beginning at time zero. Time points were recorded through 96 h.

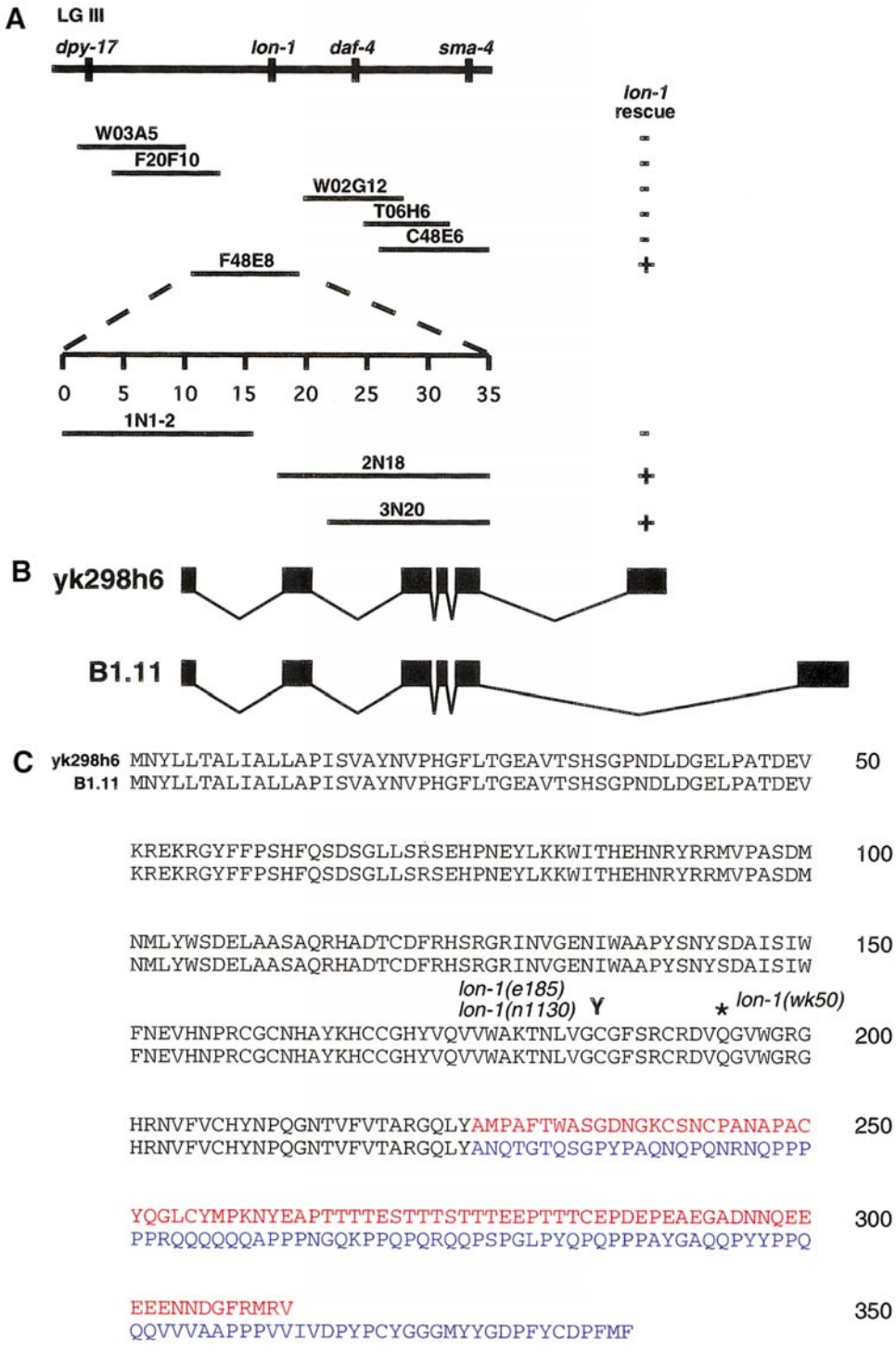


FIG. 1. *lon-1* encodes two different transcripts. (A) Genetic map of the region surrounding *lon-1*. Cosmids spanning the *lon-1* region were injected into *lon-1(e185) unc-119(e2498); him-5(1490)* animals, and two phage clones containing the *lon-1* ORF rescuing fragment were isolated (3N20, 2N18). (B) Intron/exon structure of the two *lon-1* cDNA molecules. In each cDNA, the first five exons are identical, while the last exon differs. (C) The two predicted amino acid sequences of LON-1 resulting in proteins with different C-terminal ends. In *lon-1(e185)* and *lon-1(n1130)*, a cysteine is replaced by a tyrosine, while the asterisk denotes a premature stop codon in *lon-1(wk50)* resulting in a truncated protein of 193 amino acids.

lon-1p::gfp Fusion and Tissue-Specific Expression Constructs

Sequence corresponding to 3 kb upstream of the predicted *lon-1* start site was cloned into the GFP insertion vector pPD95.69 (A. Fire, Carnegie Institute of Washington). To analyze expression patterns, this construct was coinjected with pRF4 [*rol-6(su-1006)*] (Mello *et al.*, 1991) into N2 animals. The extrachromosomal array was integrated into the genome by exposing animals to gamma irradiation and observing the F_2 population for a 100% roller phenotype (Mello and Fire, 1995). GFP fluorescence was visualized by using a Zeiss compound microscope.

Tissue-specific expression constructs were generated by cloning PCR products from the promoter regions of *rol-6*, *elt-2*, and *elt-3* into pBluescript SK+ (Gilleard *et al.*, 1999; Hawkins and McGhee, 1995; Mello *et al.*, 1991). A 1-kb PCR fragment containing a portion of the *elt-2* promoter was generated by using the primers CCATAAATTCTTGAAAGCTTTTGAT and CCGGTCTAGAGTGTATAATCTATTTTCTAGTTTCTA. A 2-kb PCR fragment containing a portion of the *elt-3* promoter was generated by using the primers CCGGAAGCTTGTGACACGTTGTTTCACGGTCAT and CCGGTCTAGAGAAGTTTGAAATACCAGGTAGCCGA. For *rol-6*, an 800-bp fragment containing a portion of its promoter was generated by using the primers CCGGAAGCTTCTTCGTATTAGATCTCAGCAGC and CCGGTCTAGACAGTTAGATCTAAAGATATATCCAG. These products were inserted into pBluescript SK+ at the *Hin*dIII and *Xba*I sites. A 4.8-kb PCR product spanning the *lon-1* genomic region was generated with the primer set CCGGGCGGCCGCCAAAATGAATTATCTGTTGACTGCTCTG and CGCGCCGCGGTTTTCATCGATATATTTGAGTTCTTTTGATT, digested with *Not*I and *Sac*II, and inserted into the constructs above containing the tissue-specific promoters. Each construct was then microinjected into *lon-1(wk50)* animals and analyzed for transformation rescue of body size. As controls, each promoter fragment described above was inserted into the promoterless *gfp* insertion vector pPD95.75 (A. Fire, Carnegie Institute of Washington) at the *Hin*dIII and *Xba*I sites, injected into *lon-1(wk50)* animals, and expression patterns were analyzed.

Northern Blots

To analyze transcriptional regulation of the *lon-1* locus, total RNA was collected from N2, *sma-6(wk7)*, *ctIs40*[*pTG96 (sur-5::gfp)*, *ZC421(dbl-1)*], and *lon-1(wk50)* L4 animals. A synchronous population was generated as described above. L4 animals were washed off plates with M9. Pelleted animals were resuspended in 100 μl Trizol (Gibco/BRL) and freeze-thawed three times. After the third thaw, samples were homogenized for approximately 5 min. Sample volumes were adjusted to 500 μl with Trizol, incubated for 5 min at room temperature, and then 100 μl of chloroform was added. The solution was shaken vigorously and centrifuged at 12,000*g* for 10 min at 4°C. The aqueous phase was precipitated with 500 μl of isopropanol and the pellet was washed with 75% ethanol.

Total RNA (20–30 μg) was loaded per lane onto a 1.2% agarose/6.6% formaldehyde gel. After gel electrophoresis, the samples were transferred to nitrocellulose (Osmonics Inc.) and baked for 2 h at 80°C. The *lon-1* probe was generated from an *Eco*RI fragment digested from the *lon-1* cDNA, B1.11. Probes were labeled by using the Prime-It II kit (Stratagene). The blot was prehybridized for at least 30 min in 1 mM EDTA, 0.5 M $NaPO_4$, pH 7.2, 7% SDS, and 1% BSA fraction V (Sigma) at 65°C. Probes were added to the prehybridization solution and incubated overnight at 65°C. Each blot was washed three times for 15 min at 65°C in a wash containing 1 mM Na_2EDTA, 40 mM $NaPO_4$, pH 7.2, and 1% SDS. After the last wash, blots were placed onto a phosphorimager screen and analyzed by using a Molecular Dynamics PhosphorImager (Amersham Biosciences).

TABLE 1

Epistatic Analysis between *lon-1* and Sma/Mab Pathway Components

Mutant	Perimeter (mm)	*n*
N2	2.44 ± 0.16	40
sma-6(wk7)	2.01 ± 0.14	42
lon-1(wk50)	2.88 ± 0.15	42
sma-6(wk7);lon-1(wk50)	2.63 ± 0.14	42

RESULTS

lon-1 Affects Body Size and Is Epistatic to Sma/Mab Pathway Components

Mutations in any of the known Sma/Mab pathway components (*dbl-1*, *daf-4*, *sma-6*, *sma-2*, *sma-3*, and *sma-4*) result in animals with body size defects and male tail abnormalities (Estevez *et al.*, 1993; Krishna *et al.*, 1999; Morita *et al.*, 1999; Savage *et al.*, 1996; Suzuki *et al.*, 1999). Body sizes are approximately 70% of the wild-type body length. Examination of Sma/Mab mutant male tails reveals that several sensory rays are fused to one another. Ray fusions occur between rays 4 and 5, rays 6 and 7, and rays 8 and 9 with a higher percentage of fusions observed between rays 6 and 7 (Savage *et al.*, 1996). In addition, the copulatory spicules are crumpled. Since TGFβ-like signaling in *C. elegans* affects body size morphogenesis, we decided to investigate body size regulation in *lon-1* mutants. Mutations in *lon-1* result in worms that are approximately 25% longer than wild-type animals. Double mutants consisting of *lon-1* and each of the following, *sma-6(wk7)*, *dbl-1(wk70)*, *sma-2(e502)*, *sma-3(wk30)*, and *sma-4(e729)*, result in worms which are longer in body size than wild-type animals, therefore placing *lon-1* downstream of the Sma/Mab pathway components (Table 1; and data not shown). Although *lon-1(lf)* suppresses the small body size phenotype seen in the Sma/Mab pathway components, it does not suppress the ray fusion defects (data not shown).

lon-1 Encodes a Protein with Homology to the CRISP Family of Proteins

In order to understand how *lon-1* functions, we undertook the molecular cloning of *lon-1*. *lon-1* was first identified and mapped to an interval on chromosome III by Brenner (1974). We have rescued the *lon-1* mutant phenotype by cosmid injection (Fig. 1A). Cosmid F48E8 not only rescued the *lon-1* phenotype, but also occasionally con-

TABLE 2
Rescue of *lon-1(wk50)* by Promoter Fusion Constructs

Genotype	Perimeter of adult (mm)	*n*
N2	2.56 ± 0.11	23
lon-1(wk50)	2.95 ± 0.13	46
lon-1(wk50);rol-6p::lon-1 (hyp6, hyp7)	2.57 ± 0.15	36
lon-1(wk50);elt-2p::lon-1 (intestine)	2.84 ± 0.27	19
lon-1(wk50);elt-3p::lon-1 (hypodermis)	2.36 ± 0.59	22
lon-1(wk50);lon-1p::lon-1	2.39 ± 0.19	30

ferred a small body size phenotype (data not shown). This suggests that proper dosage of *lon-1* is required for correct body size formation. To identify the *lon-1* open reading frame (ORF), we isolated genomic clones and found that two of these, 2N18 and 3N20, also rescued (Fig. 1A). A construct containing a single ORF common to each clone, corresponding to F48E8.1, also conferred rescue, indicating that it is the *lon-1* transcript (Table 2).

To prove that this ORF correlates with the *lon-1* transcript, we set out to identify molecular lesions in *lon-1(wk50)*, *lon-1(e185)*, and *lon-1(n1130)*. Mutant *lon-1* loci were amplified and sequenced. *lon-1(e185)* and *lon-1(n1130)* have an identical mutation, a nucleotide base change from G to A resulting in an amino acid change from cysteine to tyrosine at amino acid position 185. *lon-1(wk50)* animals have a mutation that results in a premature stop codon at amino acid position 194 and, therefore, a truncated protein (Fig. 1C). Taken together, these data confirm F48E8.1 as the *lon-1* transcript. It is likely that these nucleotide changes in the *lon-1* transcript do not result in null phenotypes. It has previously been reported that overexpression of *dbl-1* in a *lon-1(lf)* background results in animals which are longer than mutants alone (Morita *et al.*, 1999). The phenotypes in the *lon-1* alleles used in this study are not as severe as *lon-1(e185)*, suggesting that they are hypomorphic alleles.

After identifying the *lon-1* ORF, we obtained two *lon-1* cDNAs. We isolated B1.11 by screening a cDNA library (R. Barstead, personal communication) for the *lon-1* transcript. An additional cDNA, yk298h6, was obtained from the *C. elegans* cDNA project (Y. Kohara, National Institute of Genetics). Both cDNA transcripts contain six exons. However, the sixth exon differs between the two. To rule out the possibility that one of these cDNAs is an aberrant form made during library construction, PCR amplification from the λACT-RB2 (R. Barstead, personal communication) cDNA library was performed. The presence of both *lon-1* transcripts was detected, suggesting that there are two forms of *lon-1*. Each is spliced differently between the fifth and sixth exons resulting in proteins with different C-terminal ends (Fig. 1B). The two different *lon-1* transcripts, yk298h6 and B1.11, encode predicted proteins of 313 and 337 amino acids, respectively. Each protein contains a predicted secretion signal at its N terminus and a region of approximately 155 amino acids that shows homology to extracellular proteins of the CRISP superfamily. This family includes the sperm-coating glycoproteins, vespid wasp venom allergen 5 (Ag5), plant pathogenesis proteins of the PR-1 family, and human glioma pathogenesis-related protein (GliPR) (Fig. 2). Members of this family are very diverse and little is known about how they function. This family is defined by a region of several conserved cysteine residues within the carboxyl-terminal domain, as well as a hydrophobic signal sequence at the amino terminus.

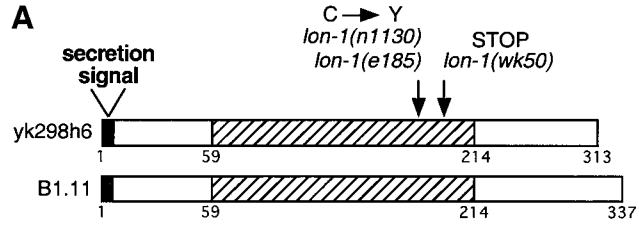
LON-1 Is Expressed in Hypodermal and Intestinal Cells

To determine where *lon-1* functions, we examined its expression patterns *in vivo*. Approximately 3 kb of the *lon-1* promoter upstream of the start site was fused to *gfp* (*lon-1p::gfp*). This construct was injected into wild-type animals and its expression pattern was examined. We see similar patterns of expression from early larval stages through the adult stage (Figs. 3A, 3C, and 3E; data not shown). In L1 hermaphrodites, expression can be seen in the intestine, with the posterior gut showing more expression than the anterior gut. In the posterior-most part of the hermaphrodite, the hypodermal tail region also expresses this reporter. Occasionally, in the head, hypodermal regions show expression along with a pair of unidentified neurons. L4 and adult animals show a similar expression pattern to that seen in L1 hermaphrodites. In addition, L4 and adult animals show expression in hypodermal regions through the entire body length of the animal. Four stripes of alternating thickness can be seen, originating in the hypodermal regions of the head and spanning the length of the animal to the posterior most hypodermal regions of the tail.

Expression constructs with Smad promoters (*sma-2p::gfp*, *sma-4p::gfp*, and *sma-3p::lacZ*) as well as the type I RSK promoter (*sma-6p:: gfp*) also show similar temporal and spatial expression patterns to those observed for *lon-1p::gfp* (Savage-Dunn *et al.*, 2000; Yoshida *et al.*, 2001; data not shown). This includes the hypodermal stripes that extend the entire body length of the animal. This result suggests that *lon-1* may function together with Smads to regulate body size development.

Hypodermal Expression Is Sufficient for Rescue of the lon-1 Phenotype

Since *lon-1p::gfp* is expressed in the hypodermal and intestinal regions of the animal, we asked whether *lon-1* functions in these tissue types as well. Promoters that drive expression in specific tissues were fused to *lon-1* genomic DNA. Tissue-specific promoters used in this study included *rol-6* (hypodermal expression), *elt-2* (intestinal expression), and *elt-3* (hypodermal expression) (Gilleard *et al.*, 1999; Hawkins and McGhee, 1995). As controls, each promoter driving GFP expression alone was analyzed for expected



B

	%I	%S
At PR1	35.1	41.8
Hs GliPR	36.6	41.8
Hs TPX-1	31.3	39.5
vw VA5	38.4	47.1
wh VA5	41.3	50.0
Dm VA5-2	29.4	36.8
Dm CG8483	46.8	54.0

C

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                                                                                                  *       *
Ce LON-1 FPSHFQSDSGLLSRSEHPNEYLKKWITHEHNRYRRMV..............PASDMNMLYWSDELAASAQRHADTCDFRHSRGRI....NV.GENI
At PR1   ....VFVALVGALVLPSKAQDSPQDYLRVHNQARGAVGVGP..................MQWDERVAAYARSYAEQL...RGNCRLI..HSG.GP..
Hs GliPR .SFVSNYSHTANILPDIENEDFIKDCVRIHNKFRSEV............KPTASDMLYMTWDPALAQIAKAWASNCQFSHNT.RLKPPHKL.HPNF
Hs TPX-1 .AEGKDPAFTALLTTQLQVQ...REIVNKHNELRKAV............SPPASNMLKMEWSREVTTNAQRWANKCTLQHS....DPEDR...KTS
ve VA5   ............VSYGLTKQEK.DILKEHNDFRQKIARGLETRGNPGPQPPAKNMKNLVWNDELAYVAQVWANQCQYGHDTCRDVAKYQV.GQNV
wh VA5   ............KAYGLTNDEKNEILKRHNDFRQNVAKGLETRGKPGPQPPAKNMNVLVWNDELAKIAQTWANQCDFNHDDCRNTAKYQV.GQNI
Dm VA5-2 ............ELIDINDDYKWVFVHSHNDKRNYIAGGYDSNHNA.....ACRMATMEWDDELAYLASLNVRQCNMVHDSCHNTDAFKYSGQNL
Dm CG8483 ......................ILQEHNRLRQIVATG....RYPG.QPGAENMREIVWDDELAARAQKWADNCQFRHDPHRTINRFTM.GQNL

                                                                                  ⊛       *               *
Ce LON-1 .........WAAPYSNYSDAISIWFNEV..HNPRC....GCNHAYKHCCGHYVQVVWAKTNLVGCGFSRCRDVQGVWGRGHRNVFVCHYNPQGN..
At PR1   ..YGENLAWGSGDLSGVS.AVNMWVSEKANYNYAA....NTCNG...VCGHYTQVVWRKSVRLGCAKVRCNN........GGTIISCNYDPRGN..
Hs GliPR TSLGENIWTGSVPIFSVSSAITNWYDEIQD..YNF....KTRICKKV.CGHYTQVVWADSYKVGCAVQFCPKVSGFDALSNGAHFICNYGPGGN..
Hs TPX-1 TRCGENLYMSSDPT.SWSSAIQSWYDEILDFVYGV....GPKSPNAV.VGHYTQLVWYSTYQVGCGIAYCPNQ...DSLK..YYYVCQYCPAGN..
ve VA5   ALTGSTAA....KVDDPVKLVKMWEDEVKDYNPKK....KFSGNDFLKTGHYTQMVWANTKEVGCG.....SIKYIQEKWHKHYLVCNYGPSGN..
wh VA5   AISSTTAT....QFDRPSKLIKQWEDEVTEFNYKV....GLQNSNFRKVGHYTQMVWGKTKEIGCG.....SIKYIPDNWYTHYLVCNYGPGGN..
Dm VA5-2 AWQAYSGDLPDMGVI.LDNSVQMWFDEVHNSNAGIIAGGYPSGYNGPAIGHFTVMMSERNTRLGCAAAR.....YNRDGWNQVLVACNYASTN...
Dm CG8483 AIIWSTAPLDADDGDFPSR.IQSWPNEVQKYSFGD....AWSP....KTGHYSQLVWGETSLVGCG.....YAEYKPTSKYNKLYVCNYGPGGNVV
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FIG. 2. *lon-1* encodes a protein with homology to members of the CRISP superfamily. (A) Schematic of LON-1. Hatched area is region of LON-1 that shows homology to members of the CRISP superfamily. (B) Percent similarities and identities of the homologous region found between LON-1 and several CRISP superfamily members: *Ce* LON-1, *C. elegans* LON-1; *At* PR1, *Arabodopsis thaliana* pathogenesis-related protein 1; *Hs* GliPR, *Homo sapiens* glioma pathogenesis-related protein; *Hs* TPX-1, *Homo sapiens* testis-specific antigen; vw VA5, vespid wasp venom allergen 5; wh VA5, white hornet venom allergen 5; *Dm* VA5–2, *Drosophila melanogaster* venom allergen 5–2; *Dm* CG8483, *D. melanogaster* predicted CRISP family member. (C) Alignment of the hatched region of LON-1 shown in (A) and the corresponding regions found within several CRISP family members. Asterisks indicate the conserved cysteines found in these proteins. The circled asterisk identifies one of the conserved cysteines mutated in *lon-1(e185)* and *lon-1(n1130)*.

tissue-specific expression (data not shown). Each construct was injected into *lon-1(wk50)* animals and multiple transgenic strains were analyzed. A representative strain is shown (Table 2). We find that *lon-1* genomic DNA under control of the hypodermal specific promoters, *rol-6* or *elt-3*, is able to rescue the long body size phenotype of *lon-1(wk50)* animals to that of wild-type body size. Quite often these constructs also conferred a small phenotype, which would be expected if *lon-1* were to function with the Sma/Mab pathway components to regulate body size morphogenesis. However, *lon-1* genomic DNA under the control of *elt-2*, the intestinal-specific promoter, did not rescue the long body size phenotype, suggesting that *lon-1* function is dispensable in the intestine for regulating body size.

lon-1 Animals Differ in Body Length Compared with Wild-Type at Later Developmental Stages

Previously, it has been demonstrated that Sma/Mab mutant animals are the same length as wild-type animals at hatching (Savage-Dunn *et al.*, 2000); L1 larvae of *sma-2*, *sma-3*, *sma-6*, and *dbl-1* are indistinguishable in length from N2 larvae. As the Sma/Mab pathway mutant animals develop postembryonically, they are approximately 70% the size of wild-type animals 96 h after hatching (Savage-Dunn *et al.*, 2000). We wondered whether *lon-1* mutant animals develop in a manner comparable to the Sma/Mab pathway mutant animals. Are *lon-1* animals longer in body length than wild-type animals at hatching, or are the L1 larvae identical in body length to that of wild-type animals? We find that the L1 larvae of *lon-1(e185)*, *lon-1(wk50)*, *lon-1(n1130)*, *lon-1(ct411)*, and *lon-1(wk49)* are indistinguishable from wild-type and Sma/Mab mutant L1 larvae (Fig. 4; and unpublished data). This suggests that the defects seen in the altered body morphology of *lon-1* mutant animals, like those of Sma/Mab mutant animals, are a result of postembryonic events. In contrast, *sma-1*, which is not a Sma/Mab pathway component, produces L1 larvae that are approximately half the size of wild-type animals (McKeown *et al.*, 1998). We also see that the *lon-1* mutants gradually get longer in body length compared with wild-type animals as they pass through the remaining larval stages and eventually become adults.

The Sma/Mab Pathway Regulates lon-1 Expression

In various organisms, TGFβ signaling regulates the transcriptional activity of many genes. However, very few

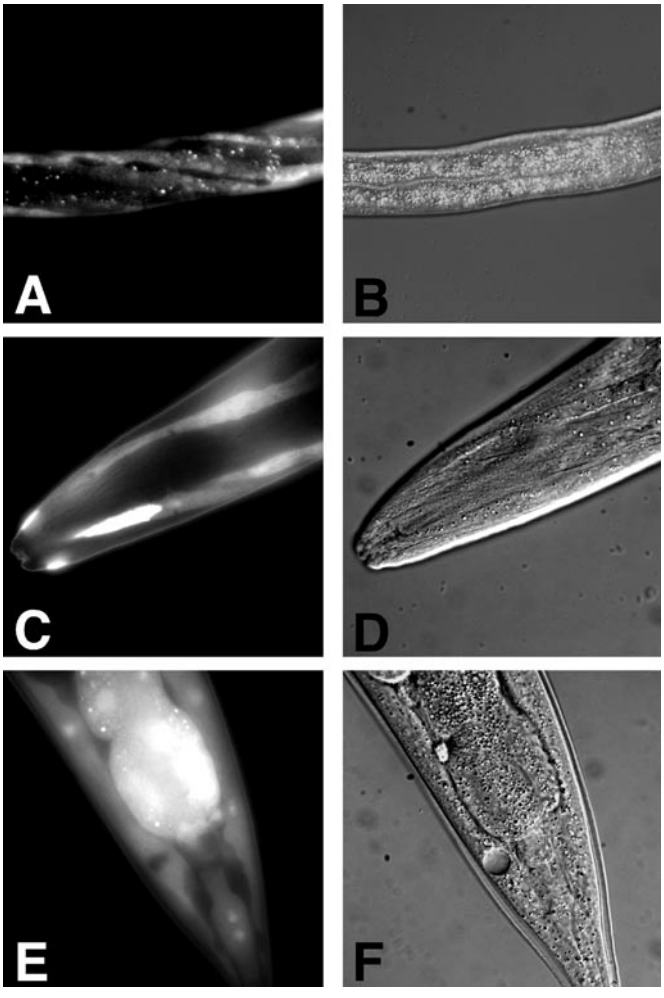

FIG. 3. Expression patterns of *lon-1p::gfp* in wild-type animals. Fluorescent images (A, C, E) are on the left, while Nomarski images (B, D, F) of the same animals are on the right. Hypodermal regions express *lon-1p::gfp* in a striped pattern down the entire length of the animal in L1 (not shown), L4 (A), and adult (C) animals. Expression is also observed in the anterior and posterior regions of the intestine in L4 animals (data not shown) and adults (E).

downstream target genes regulated by the *C. elegans* Sma/Mab pathway have been characterized. Since *lon-1* is epistatic to known Sma/Mab pathway signaling components (Table 1), we wondered whether Sma/Mab pathway signaling might negatively regulate *lon-1* mRNA expression levels. Total RNA from N2, *sma-6(wk7)*, *ctIs40*[*pTG96 (sur-5::gfp), ZC421(dbl-1)*], and *lon-1(wk50)* animals was isolated from synchronous populations of L4 animals and used for Northern blot analyses. The *sma-6(wk7)*-null allele was used to provide RNA from an animal where Sma/Mab pathway signaling is lost. In *ctIs40*[*pTG96 (sur-5::gfp), ZC421(dbl-1)*] animals, *dbl-1* is overexpressed, which leads to an up-regulation of pathway signaling. We observe that *lon-1* mRNA levels are up-regulated in *sma-6(wk7)* and down-regulated in *ctIs40*[*pTG96 (sur-5::gfp), ZC421(dbl-1)*]; quantitation shows a difference of approximately 45%. This difference is reproducible and consistent with negative regulation of the *lon-1* message by the Sma/Mab pathway (Fig. 5, lanes 3 and 2, respectively).

DISCUSSION

lon-1 Regulates Body Size

Although the Sma/Mab pathway regulates body size formation, little is understood about the molecular mechanism of this regulation. The Sma/Mab pathway components are expressed in various tissues throughout development. The ligand *dbl-1* is expressed mostly in neurons found in the ventral nerve cord and pharynx, while the type I RSK, *sma-6*, is expressed in pharyngeal muscles, intestinal cells, and hypodermal tissues (Krishna *et al.*, 1999; Suzuki *et al.*, 1999). *daf-4*, the type II RSK, partially functions in pharyngeal, muscular, and hypodermal tissues to regulate body size (Inoue and Thomas, 2000). The Smads *sma-2*, *sma-3*, and *sma-4* are also expressed in hypodermal tissues, further implicating the hypodermis as the site responsible for regulation of body size (unpublished data; Savage-Dunn *et al.*, 2000). Our work presented here provides compelling evidence that body size is regulated in the hypodermal tissues of *C. elegans*. We see that *lon-1*, when expressed specifically in hypodermal tissues under the control of the *rol-6* or *elt-3* promoters, is able to rescue the Lon phenotype of *lon-1(wk50)* animals to that of wild-type, while *lon-1* expression under the control of the intestinal promoter *elt-2* has no effect on body size (Table 2). Although expression of *lon-1(+)* in the intestine has no effect on body size, *lon-1* is expressed in the intestine, suggesting that it may play a yet to be identified role in the intestine.

The Sma/Mab Pathway Branches Downstream of the Smads

Mutations in any of the Sma/Mab pathway components, *dbl-1* (ligand), *sma-6* (RSK I), *daf-4* (RSK II), *sma-2* (Smad), *sma-3* (Smad), or *sma-4* (Smad), result in animals that are about 70% the size of wild-type animals (Estevez *et al.*, 1993; Krishna *et al.*, 1999; Morita *et al.*, 1999; Savage *et al.*, 1996; Suzuki *et al.*, 1999). In addition, male mutant animals have defects in their tail rays and spicules. Double mutants made between any of the Sma/Mab pathway components and *lon-1* result in animals that are Lon, indicating that *lon-1* is epistatic to Sma/Mab pathway signaling. Although *lon-1* is able to suppress the small body size phenotype, it is unable to rescue the male tail defects also found in the Sma/Mab pathway mutants (unpublished data). This suggests that the Sma/Mab pathway may branch downstream of Smad (*sma-2*, *-3*, and *-4*) activity. Once the Smads translocate into the nucleus, they would then be able to regulate a variety of genes. A subset of these genes may only

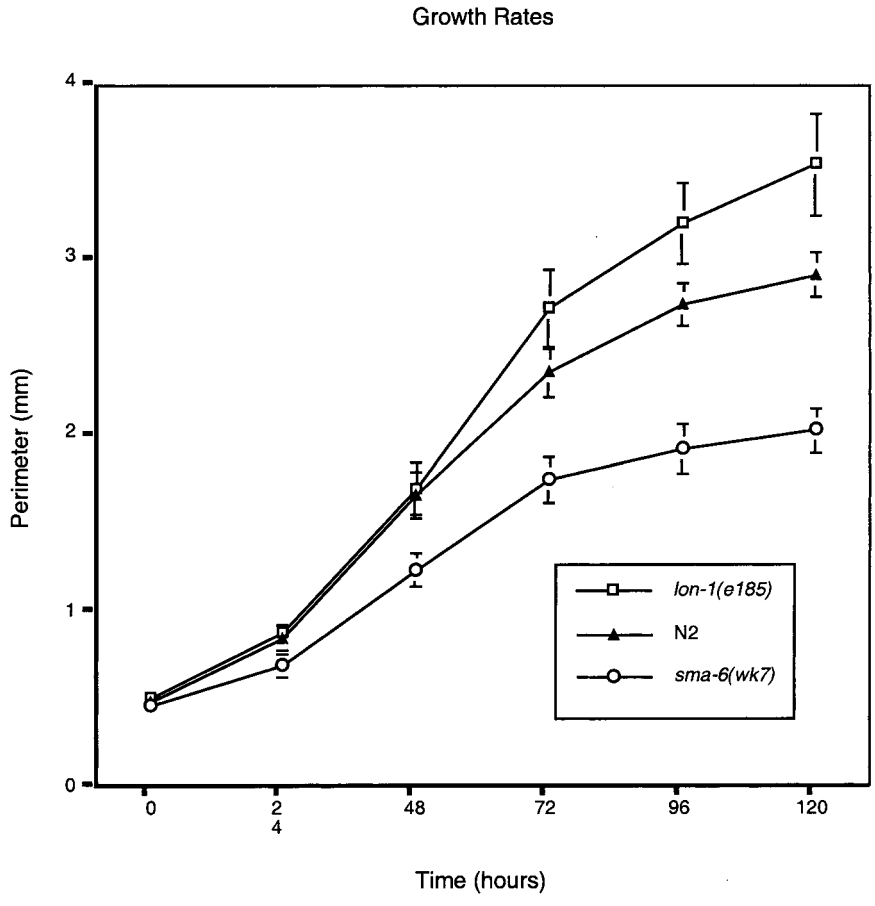

FIG. 4. *lon-1* animals are longer in length than wild-type animals at later developmental stages. *lon-1(e185)*, *sma-6(wk7)*, and N2 animals were synchronized and measured at 24-h time points starting with L1 larvae at time zero. All animals are indistinguishable in length as L1 larvae. Over time, Lon animals gradually develop a longer body length than wild-type animals. The perimeter of at least 30 animals for each strain was measured and averaged at each time point.

affect body size formation, while others might only regulate male tail morphogenesis. Further evidence in support of pathway branching is observed in a weak allele of *sma-6*, where the small body size phenotype is observed without defects in male tail rays, thus arguing that body size morphogenesis and male tail formation are separable functions (Krishna *et al.*, 1999). Alternatively, *lon-1* might not suppress the tail defects seen in Sma/Mab pathway mutants because the alleles used in this study may not be null mutations. Previously, it has been shown that overexpression of *dbl-1* in the *lon-1(e185)* background results in animals which are longer than *lon-1(e185)* animals alone, suggesting that *lon-1(e185)* is not null (Morita *et al.*, 1999). Although the *lon-1(wk50)* lesion is likely to result in a truncated protein, the Lon phenotype seen in these animals is less severe than that seen in *lon-1(e185)* animals and suggests that the truncated protein maintains partial function.

Negative Regulation of the lon-1 Transcript Affects Body Size Formation

Because defects in Sma/Mab pathway signaling and mutations in *lon-1* affect body size, we reasoned that *lon-1* might somehow be involved in regulating body size in conjunction with the Sma/Mab pathway components. Double mutants made between the null allele of *sma-6* and *lon-1* are Lon, therefore eliminating the possibility that *lon-1* acts as a negative regulator of the pathway. This evidence led us to hypothesize that *lon-1* is negatively regulated by the Sma/Mab signaling pathway. In *sma-6(wk7)*-null mutants, the Smads *sma-2*, *sma-3*, and *sma-4* would not be activated, would not translocate to the nucleus, and would not bind the *lon-1* promoter. Therefore, the *lon-1* message would become up-regulated in the absence of the Smads. The absence of Smad binding might allow for other molecules to bind to and positively regulate the *lon-1* promoter. Conversely, excess ligand may over-

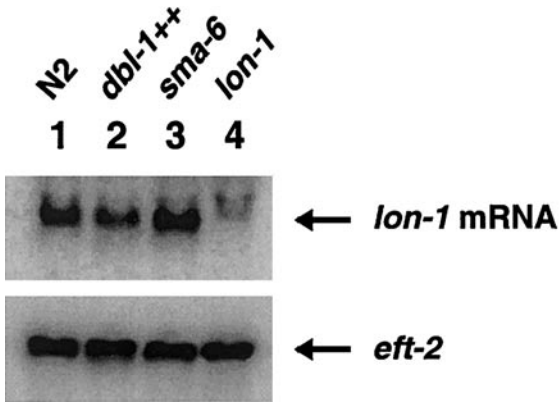

FIG. 5. *lon-1* mRNA levels are negatively regulated by Sma/Mab pathway signaling. Northern blot showing *lon-1* mRNA transcripts found in total RNA isolated from N2 (lane 1), *dbl-1++* (*ctIs40*; see Materials and Methods) (lane 2), *sma-6(wk7)* (lane 3), and *lon-1(wk50)* (lane 4) L4 animals. *lon-1* transcripts are up-regulated in *sma-6(wk7)* animals where Sma/Mab pathway signaling is greatly reduced (lane 3). However, in *dbl-1++* animals, which overexpress *dbl-1* (ligand) and increase pathway signaling, *lon-1* mRNA is reduced (lane 2). *lon-1* mRNA from *lon-1(wk50)* animals is minimal (lane 4). Elongation factor-2 (*eft-2*) was used as a control to measure the amount of total RNA loaded into each lane. mRNA amounts were quantitated by using a phosphorimager.

stimulates the pathway, resulting in increased RSK activity. This increased activity would signal more Smads into the nucleus, which in turn would bind the *lon-1* promoter and decrease the expression levels of the *lon-1* message.

In fact, we observe that the *lon-1* message is up-regulated in *sma-6(wk7)* animals, and is down-regulated in *ctIs40*[*pTG96(sur-5::gfp), ZC421(dbl-1)*] animals where *dbl-1* ligand is overexpressed. The difference seen in *lon-1* mRNA levels between the animals lacking *sma-6(wk7)* and those overexpressing *dbl-1* is approximately 45%. It is likely that the transcriptional control of the *lon-1* message is complex. It is quite possible that there are other regulatory molecules in addition to the Smads that modulate *lon-1* expression. Expression of *lon-1* mRNA is still observed in animals overexpressing *dbl-1* (*ctIs40*[*pTG96(sur-5::gfp), ZC421(dbl-1)*]), indicating that the regulation of *lon-1* mRNA levels is not a simple ON/OFF mechanism. It is probable that wild-type body size is maintained by proper dosage of LON-1, and either too much or too little *lon-1* mRNA results in small or long body sized animals, respectively.

Smads have been shown to bind DNA in a sequence-specific manner. Human Smad3 and Smad4, *Drosophila* Mad, and *C. elegans* DAF-3 bind to specific consensus sites in various TGFβ responsive promoters (Kim *et al.,* 1997; Thatcher *et al.,* 1999; Zawel *et al.,* 1998). Examination of the *lon-1* promoter sequence reveals three well-conserved Smad binding sites upstream of the *lon-1* ATG. Each of these binding sites shows high homology to the sites bound by human Smad3 and 4, *Drosophila* Mad, and *C. elegans* DAF-3. The presence of putative Smad binding sites in the *lon-1* promoter provides a possible molecular explanation for our findings that *lon-1* is transcriptionally regulated by the Sma/Mab pathway.

lon-1 Encodes Two Different Transcripts

We have identified two different *lon-1* cDNA clones from independent cDNA libraries. Both cDNAs were also amplified from a third independent cDNA library, supporting the existence of two *lon-1* mRNAs. Each cDNA contains six exons; one through five are identical, while the sixth differs. The different C-terminal domains found in each protein are likely to determine their functional roles. Whether both of these cDNA forms are required for proper body size formation remains to be determined. The C-terminal domain of LON-1 may contain some sort of regulatory domain, found in one protein and not the other, needed for proper functioning relative to body size morphogenesis. It is important to remember that *lon-1* expression is observed in the intestinal tissues but is not required for body size formation. Therefore, one or both transcripts may also play a role in the intestine.

LON-1 Is a Member of the CRISP Superfamily

lon-1 encodes a protein with sequence similarity to a diverse family of proteins referred to as the CRISP superfamily; these proteins are secreted, and possess several conserved cysteine residues (Kratzschmar *et al.,* 1996). This family consists of numerous proteins, including the human GliPR protein, antigen 5, testes-specific proteins, and several plant pathogenesis-related (PR) proteins. The human GliPR protein is highly expressed in the tumor glioblastoma multiforme, but not in normal fetal or adult brain tissues (Murphy *et al.,* 1995). These tumors derive from immunological tissues and comprise more than half of the human primary tumors identified in the brain (Morris and Schoene, 1984). GliPR protein possesses a high degree of identity (37–42%) with the plant PR-1 proteins, which have been shown to be important in mechanisms involved in plant defense systems (Bol *et al.,* 1990; Murphy *et al.,* 1995). In addition, antigen 5 from vespid wasp venom and testes-specific proteins are structurally related to the PR-1 proteins, but to a lesser extent than the GliPR protein (Charest *et al.,* 1988; Kasahara *et al.,* 1989; King *et al.,* 1990; Lu *et al.,* 1993; Murphy *et al.,* 1995). TPX-1 (also identified as CRISP-2) is a testes-specific protein involved in the differentiation of spermatogenic cells (Maeda *et al.,* 1998). While it is clear that these proteins share functional modules that are utilized to regulate a wide variety of biological processes, a mechanistic explanation for the specific molecular role of these domains remains elusive. How *C. elegans* LON-1 functions in relation to these proteins remains to be determined. It is possible that LON-1 is secreted from the hypodermal cells and functions as a ligand for another signaling pathway, or as a matrix scaffolding protein. A

Drosophila homolog has also been identified; however, mutants have yet to be described. Once characterized, any *Drosophila* mutations may provide additional insights into LON-1 function. We have performed genetic screens to suppress the *lon-1* phenotype in *C. elegans* and have identified small mutant animals. Cloning and characterization of these genes may help in determining the role *lon-1* plays in *C. elegans* body size morphogenesis.

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